EXHIBIT A

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The Characteristics of Inhibition of Protein Synthesis by Double-Stranded Ribonucleic Acid in Reticulocyte Lysates*

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SUMMARY

All types of double-stranded RNA (dsRNA) tested inhibit protein synthesis in rabbit reticulocyte lysates. The inhibition is characterized by its strongly biphasic kinetics, and can be enhanced by preincubation of the lysate with dsRNA in the absence of protein synthesis. Only properly and extensively matched dsRNA (greater than about 50 base pairs) has this property; no form of DNA, single-stranded RNA or even RNA-DNA hybrids act as inhibitors in this way. The cause of the inhibition appears to be a failure of initiator tRNA to associate with native ribosomal subunits in the initiation process (DARNBROUGH, C., HUNT, T., AND JACKSON, R. J. (1973) *Biochem. Biophys. Res. Commun.* 48, 1556–1564). We have shown that this block is not accompanied by stable association of dsRNA with the ribosomes. There are several reasons to believe that the mechanism of action of dsRNA may be complex with the possible involvement of at least one catalytic step. First, the lysate is inhibited by levels of dsRNA at which ribosomes are present in 100-fold excess over base pairs of dsRNA present. Second, high concentrations of dsRNA (greater than 10 μg per ml) are not inhibitory, but can in some, but not all experiments, reverse the inhibition caused by lower levels of dsRNA. Third, a lysate which has been inhibited by dsRNA, when mixed with a fresh lysate will inhibit synthesis in the mixture much more severely than would be expected from the concentration of dsRNA now present. These results indicate that low levels of dsRNA promote the formation of an inhibitor which may exist in two forms: one that is reversible by high levels of dsRNA and one that is irreversible.

Double-stranded RNA is an extremely potent inhibitor of the initiation of protein synthesis in reticulocyte lysates (1, 2). This effect is not a peculiarity of cell-free systems from reticulocytes, since other mammalian cell-free extracts are also inhibited by dsRNA,[1] albeit at somewhat higher concentrations (3, 4). This property of dsRNA is interesting not only for its obvious relevance in the mechanism of initiation of protein synthesis in mammalian cells, but also for its possible relevance to the numerous other biological effects of dsRNA: the induction of interferon (5, 6), enhancement of the immune response (7), the causation of tumor regression (8), and general cytotoxicity (9–11).

In this paper we describe experiments designed to elucidate the mechanism of action of dsRNA on the reticulocyte lysate. We have already shown that the inhibition of protein synthesis is characterized by loss of 40 S/Met-tRNA$_f^{Met}$ initiation complexes (12) and that the inhibition can be reversed by 3′:5′-cAMP and a variety of adenine derivatives (13). We now present evidence that although this form of inhibition is highly specific for dsRNA (although without regard to its composition or sequence of bases), dsRNA is not the proximate cause of the inhibition. We suggest that dsRNA acts by promoting the formation of an agent which probably inhibits protein synthesis by a catalytic mechanism. This agent may be closely related to that which inhibits globin synthesis in the absence of added hemin (14, 15).

MATERIALS AND METHODS

Preparation of Reticulocytes

Rabbit reticulocytes were obtained as described by Darnbrough *et al.* (16).

Preparation of Lysates

Lysates were prepared from washed reticulocytes as described by Hunt *et al.* (17) and stored frozen as lysates from individual rabbits in liquid nitrogen.

Incubation Conditions

Lysates were incubated at 30° together with a one-fifth volume of a master mix containing the components necessary for protein synthesis as previously described (16). DL-[1-^{14}C]Phenylalanine

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This work was carried out while T. Hunter was a Research Fellow of Christ's College, Cambridge; T. Hunt, a research fellow of Clare College, Cambridge; and H. D. Robertson, a Helen Hay Whitney Foundation Fellow.

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[1] The abbreviations used are: dsRNA, double-stranded RNA; dsDNA, double-stranded DNA; ssRNA, single-stranded RNA; ssDNA, single-stranded DNA; RE, replicative ensemble.

was used at a final concentration of 1.0 μCi per ml, while [^{35}S]-methionine was used at 75 μCi per ml. Samples withdrawn from incubations were treated as described (17) for estimation of acid-precipitable alkali-stable radioactivity. In cases where pre-incubation was carried out, everything except the hemin was omitted from the incubation. All incubations were carried out with [^{14}C]phenylalanine unless otherwise stated.

Preparation of Polynucleotides

RNAs from RNA Bacteriophages

f2—RNA was prepared by phenol extractions from f2 phage grown and purified as described by Engelhardt *et al.* (18). Pure 28 S phage RNA was prepared by fractionation of total f2 RNA on sucrose density gradients (19) and is termed f2 ssRNA. Alternatively, preparations, which were essentially identical with f2 ssRNA, could be prepared by treatment of f2 RNA with RNase III (see below). f2 dsRNA is defined as f2 replicative ensemble (or RE) (18) from which ssRNA tails have been removed by mild pancreatic RNase digestion as described below.

High specific activity ^{32}P-labeled f2 bacteriophage were grown according to Nichols and Robertson (20). Unlabeled f2 bacteriophage were the gift of Dr. R. E. Webster, The Rockefeller University, New York. The RE fraction from *Escherichia coli* cells infected with f2 was grown and purified according to Engelhardt *et al.* (18) and Robertson and Lodish (21). This material is a mixture of all structures which contain dsRNA, whether or not they also contain the characteristic single-stranded "tails" or nascent viral strands formed during viral replication (20, 23). Enzymatic and chromatographic analysis of the RE preparation used in these experiments performed as described (18) revealed that this unlabeled RE fraction contained about 60% dsRNA, the remainder consisting of single-stranded "tails."

f2 dsRNA labeled with ^{32}P was prepared by an adaptation of the annealing method described by Robertson and Zinder (23). ^{32}P-Labeled f2 ssRNA (specific activity 1.6×10^6 cpm/μg) (20 μg) was mixed with 60 μg of unlabeled f2 RE and boiled for 3 min in 1 ml of distilled water. The mixture was then made 0.05 M in Tris-HCl (pH 7.0), 0.1 M in NaCl, and 0.001 M in EDTA (Buffer A) and heated for another 3 hours. The material was made 35% in ethanol and layered onto a cellulose CF11 column (20, 23). On such a column, DNA and small molecular weight material elute in Buffer A containing 35% ethanol; ssRNA, in 15% ethanol; and dsRNA (plus any stably attached ssRNA), in Buffer A alone. Whereas, greater than 99% of the ^{32}P-labeled f2 RNA eluted from the column in 15% ethanol before annealing, 25% eluted in 15% ethanol and 75% in Buffer A alone after annealing. In other words, 75% of the single strands had supplanted the unlabeled "plus" strands in the double-stranded complex.

Since some of the ^{32}P-labeled material would be expected to have unlabeled "tails," the material chromatographing as dsRNA was treated with pancreatic RNase to remove remaining single strands as follows: 1 μg of Worthington pancreatic RNase was added to 1 ml of ^{32}P-labeled material in Buffer A, and incubated at 37°. After 5 min 50.5% of the RNA was solubilized, and only an additional 3.7% in the next 10 min. dsRNA shows complete resistance to pancreatic RNase digestion under these salt conditions (23). Digestion was terminated with 0.7 ml of Buffer A-saturated phenol. After extraction and centrifugation to separate the layers, the process was repeated twice more; between the second and the third extraction, the aqueous layer was made 0.1% in diethyl pyrocarbonate and incubated for 15 min at 25°. Following the final phenol extraction, the aqueous layer was made 35% in ethanol and layered onto another cellulose CF11 column. Fifty-three per cent of the material flowed through the column in 35% ethanol (representing the small molecular weight fragments from the digested single strands), while 4% remained attached to the column in 35% and 15% ethanol, eluting in Buffer A alone. This material was greater than 95% resistant to pancreatic RNase digestion in Buffer A, but completely sensitive in distilled water. This behavior is characteristic of f2 dsRNA (23). In addition, over 96% of this material was solubilized by *E. coli* RNase III, which is specific for the digestion of dsRNA (24). The final specific activity was 2.4×10^5 cpm per μg.

R17—RNA was isolated by phenol extraction from R17 phage which was the generous gift of Dr. M. Osborn, M. R. C. Laboratory of Molecular Biology, Cambridge.

Qβ—RNA was isolated by phenol extraction from Qβ phage grown and purified as described by Robertson *et al.* (24).

Nucleic Acids from Bacteriophage f1

Unlabeled f1 bacteriophage and double-stranded RF DNA were the kind gift of Dr. P. Model, The Rockefeller University, New York. During extensive synthesis of DNA-RNA hybrids and ssRNA by *E. coli* RNA polymerase with f1 ssDNA as template, dsRNA is synthesized as a side product (25). In a 10-μl reaction in 0.02 M Tris-HCl (pH 7.9), 0.0001 M dithiothreitol, 0.001 M EDTA, 0.01 M magnesium acetate were placed 0.4 μg of f1 ssDNA; 10 nmoles each of unlabeled UTP and GTP; 0.5 nmoles of [α-^{32}P]-CTP (the kind gift of Dr. G. H. Dixon, M. R. C. Laboratory of Molecular Biology, Cambridge, specific activity 6000 mCi per mmole); 2 nmoles of [α-^{32}P]ATP (specific activity 900 mCi per mmole); 2 μg of *E. coli* RNA polymerase (prepared according to Burgess (26); the kind gift of Dr. A. A. Travers, M. R. C. Laboratory of Molecular Biology, Cambridge). After 3 hours of incubation, precipitation of an aliquot of the reaction in 5% trichloracetic acid revealed that a 2.2-fold excess of RNA over input DNA had been synthesized. The various polynucleotides present in the mixture were separated and purified as follows.

DNA-RNA hybrid—An aliquot of the RNA polymerase reaction was extracted with phenol and the aqueous layer was placed onto a 5 to 20% sucrose gradient and centrifuged as described (25). The 18 S peak containing DNA-RNA hybrids was further purified by Cs_2SO_4 equilibrium density gradient as described by Colby *et al.* (27).

Double-stranded RNA—An aliquot of the RNA polymerase reaction was diluted to 0.1 ml in 0.01 M Tris-HCl (pH 7.4), 0.01 M magnesium acetate, 0.1 M NH_4Cl and treated with 25 μg per ml of pancreatic DNase (Worthington DPFF) for 15 min at 25°: then the mixture was made 2 times in Buffer A (by the addition of 0.02 ml of 10 times Buffer A) and incubated 25 min more in the presence of 5 μg per ml of pancreatic RNase. Then 1 μl of diethylpyrocarbonate was added and the reaction was diluted to 0.5 ml and extracted with 0.2 ml of phenol. The aqueous layer was made 35% in ethanol and chromatographed on a cellulose CF11 column. The vast majority of the material flowed through the column in 35% ethanol, indicating that it had been solubilized by pancreatic RNase and was therefore either free ssRNA or RNA released from the DNA-RNA hybrids by pancreatic DNase. However, 2 to 3% of the RNA chromatographed in the position of dsRNA (eluting in Buffer A alone). This RNA was pooled and its double-stranded properties were confirmed by tests with pancreatic RNase and *E. coli* RNase III. The final specific activity was 1.2×10^7 cpm per μg. The f1 dsRNA was extensively dialyzed against water and lyophilized.

Synthetic Polynucleotides

Poly(U) was obtained from British Drug Houses. Poly(A), poly(I) (which was always boiled in solution for 2 min before use), poly(A)·(U) and poly(I)·(C) were all obtained from Miles Laboratories. Poly(C) was obtained from Boehringer and Soehne. Poly(A-U) and poly(G)·(C) were prepared as described by Robertson *et al.* (24).

Other Polynucleotides

dsRNA obtained from a virus grown in *Penicillium chrysogenum* was the kind gift of Dr. D. Planterose, Beecham Research Laboratories, Betchworth, Surrey, England. Reovirus was a gift of Dr. J. J. Skehel, National Institute for Medical Research, Mill Hill, London. TMV virus was the kind gift of Dr. R. N. Perham, Department of Biochemistry, University of Cambridge. Calf thymus DNA and total yeast RNA were obtained from British Drug Houses. *Escherichia coli* ribosomal RNA was prepared from ribosomes purified from *E. coli* MRE 600 by phenol extraction. Sendai RNA was a gift of Dr. B. Mahy, Department of Virology, University of Cambridge.

Enzymes and Enzyme Assays

RNase III

RNase III was prepared as described by Robertson *et al.* (24) with the modifications described by Robertson and Hunter (19).

The conditions for digestion of nucleic acids with RNase III have been reported previously (24).

DNase

DNase was obtained from Worthington Biochemical Corp. and was DPFF grade. Conditions used for DNase digestion are described above.

Sucrose Gradients

Sucrose gradients were run as described in the appropriate figure legends and were fractionated through a flow cell which was monitored at 260 nm. Gradient fractions were precipitated with cold 8% trichloroacetic acid together with the addition of 200 μg of carrier serum albumin for estimation of radioactivity.

Materials

ATP, GTP, creatine phosphate and creatine phosphokinase were all obtained from Boehringer and Soehne, Mannheim. Hemin was obtained from Eastman Kodak. DL-[1-^{14}C]phenylalanine (50 mCi per mmole), L-[^{35}S]methionine (175 Ci per mmole) and [α-^{32}P]ATP (900 mCi per mmole) were all obtained from Amersham Radiochemical Centre.

TABLE I

Classification of nucleic acids with respect to inhibitory activity

Incubations were set up under the conditions given under "Incubation Conditions." Predigestion with RNase III was carried out as in Fig. 6 ((+) = loss of inhibition; (−) = no loss of inhibition). Preincubation with nucleic acids was carried out as in Fig. 4 ((+) = potentiation of inhibition; (−) = no increase in inhibition). With the nucleic acids in Class C no inhibition was observed up to the maximum concentration tested.

Nucleic acid	Minimum concentration to give detectable inhibition (without preincubation)	Concentration to give maximum inhibition	Sensitivity of inhibition to predigestion with RNase III	Potentiation of inhibition on preincubation with lysate	Maximum concentration tested
	ng/ml				*ng/ml*
Class A					
poly(A-U)	ND[a]	10 to 100	ND	+	
poly(A)·(U)	ND	10 to 100	ND	ND	
poly(G)·(C)	2	50 to 100	ND	+	
poly(I)·(C)	ND	10 to 100	ND	ND	
reovirus RNA	ND	10 to 100	ND	+	
f2 dsRNA[b]	1	10 to 100	+	+	
f1 dsRNA[b]	1	ND	+	+	
Penicillium chrysogenum dsRNA	1	10 to 100	+	+	
Sendai RNA	ND	10 to 100	ND	+	
Qβ RNA[b]	20	ND	ND	+	
f2 RNA[b]	10^3	ND	+	+	
R17 RNA[b]	5×10^4	ND	ND	+	
Class B					
poly(U)	10^4	ND	−	−	
poly(C)	5×10^4	ND	ND	ND	
poly(A)	10^4	ND	ND	ND	
poly(I)	10^4	ND	ND	−	
f2 ssRNA[b]	5×10^4	ND	−	−	
TMV RNA	5×10^4	ND	−	−	
Class C					
f1 hybrid[b]				−	See text
f1 ssDNA[b]				−	10^4
f1 RF DNA[b]				−	10^3
Calf thymus DNA (native)				ND	10^5
Escherichia coli rRNA				−	3×10^5
yeast RNA				ND	10^5
T4 DNA				ND	10^5

[a] ND, Not done.

[b] See "Preparation of Polynucleotides."

RESULTS

Kinetics of Protein Synthesis—Protein synthesis in rabbit reticulocyte lysates in the presence of poliovirus dsRNA exhibits two distinct phases (2). In the first, latent phase, the initial rate of protein synthesis remains unaffected: an abrupt cessation of protein synthesis marks the onset of the second, inhibitory phase. As can be seen from Table I a wide variety of dsRNAs are capable of causing inhibitions with these characteristics. A typical example of the kinetics of protein synthesis in a rabbit reticulocyte lysate incubated with hemin and f2 dsRNA (see "Preparation of Polynucleotides") is shown in Fig. 1. The level of incorporation achieved in the presence of sodium fluoride, which blocks initiation, indicates that the onset of the second phase occurs only after several complete rounds of protein synthesis have occurred (Fig. 1). In contrast the inhibition of protein synthesis obtained with single-stranded polynucleotides such as poly(U) or poly(I) is totally different in kind. In this case the initial rate of protein synthesis was depressed and there was no latent phase (Fig. 1). Also the decrease in the initial rate was roughly proportional to the concentration of polynucleotide used and the lowest concentration of polynucleotide showing inhibition was at least four orders of magnitude greater than the lowest concentrations of dsRNA showing inhibition.

Maximum inhibition by dsRNA was observed in the range 10 to 100 ng per ml, whereas 0.5 ng per ml was the lowest concentration at which inhibition was detectable unless preincubation was carried out (see below). Decreasing the inhibitor concentration from the maximally inhibitory level led to an increase in the length of the latent phase (see Fig. 2*A*). A puzzling feature of the concentration dependence is that an increase in the dsRNA concentration above the maximally inhibitory level also led to an increasing delay in the onset of inhibition (Fig. 2*B*), so much so that at the highest concentrations tested (above 10 μg per ml) there was barely any detectable inhibition. When we examined the concentration dependence of inhibition with a number of

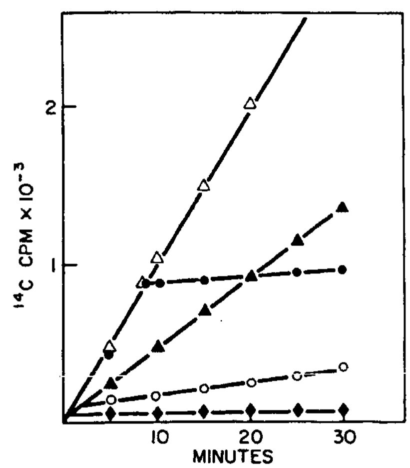
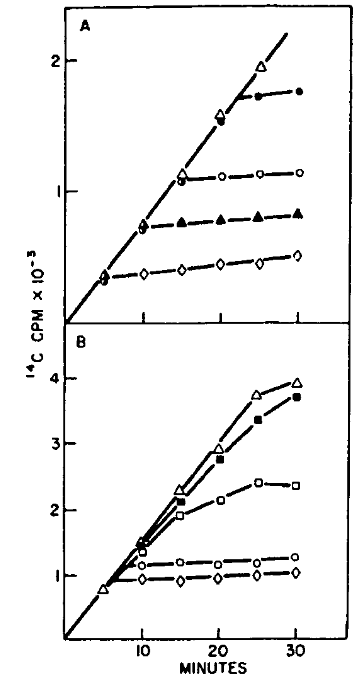


FIG. 1. Kinetics of protein synthesis in the presence of f2 dsRNA or poly(I). Samples (15 μl) were taken at the times indicated from 100-μl reactions set up and assayed as described under "Incubation Conditions." All polynucleotides and the sodium fluoride were added at the start of the incubation. △——△, control; ●——●, + 50 ng per ml of f2 dsRNA; ▲——▲, + 10^4 ng per ml of poly(I); ○——○, + 3×10^4 ng per ml of poly(I); ◆——◆, + 10 mM sodium fluoride.



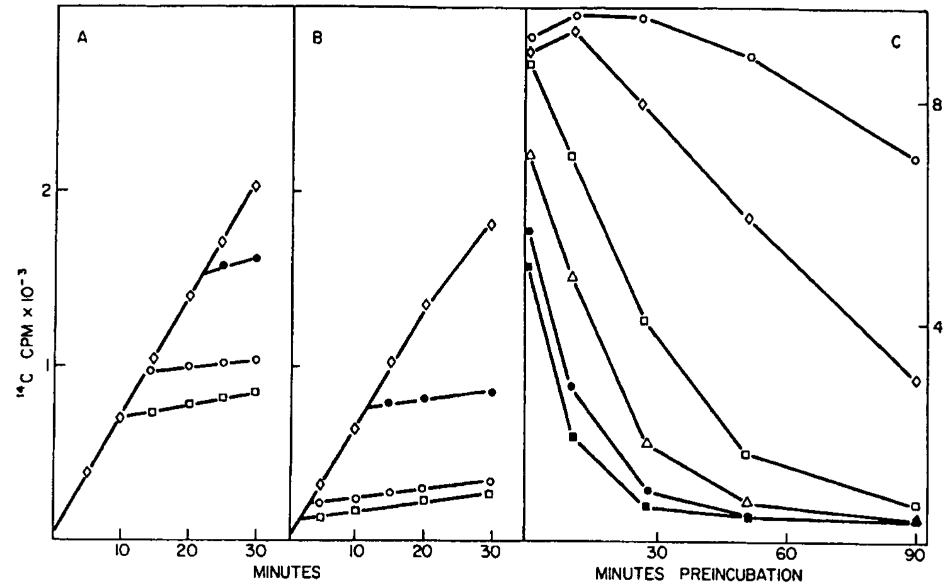


FIG. 2 (*left*). The effect of different concentrations of *Penicillium chrysogenum* dsRNA on the kinetics of protein synthesis. Samples (15 μl) were taken at the times shown from 100-μl incubations set up and assayed as described under "Incubation Conditions," except that hemin was omitted where indicated. Two different lysates were used for the experiments shown in *A* and *B*. (*A*) △——△, control; ●——●, + 0.5 ng per ml of dsRNA; ○——○, + 5 ng per ml of dsRNA; ▲——▲, + 50 ng per ml of dsRNA; ◇——◇, minus heme. (*B*) △——△, control; ■——■, + 2 × 10^4 ng per ml of dsRNA; □——□, + 2 × 10^3 ng per ml of dsRNA; ○——○, + 2 × 10^2 ng per ml of dsRNA; ◇——◇, + 20 ng per ml of dsRNA.

FIG. 3 (*right*). Effect of preincubation upon inhibition by f2 dsRNA. *A*, 100-μl incubations were set up as described under "Incubation Conditions" and f2 dsRNA was added at the concentrations shown at the start of the incubation. Samples (15 μl) were taken at the times indicated for assay of radioactivity. ◇——◇, control; □——□, + 50 ng per ml of dsRNA; ○——○, + 5 ng per ml of dsRNA; ●——●, + 0.5 ng per ml of dsRNA. *B*, 80-μl aliquots of the same lysate used for the experiment in *A* were preincubated with hemin at 30° for 30 min in the presence of the same concentrations of dsRNA used in *A*. After the preincubation protein synthesis was started by addition of 20 μl of master mix (see "Incubation Conditions") and 15-μl samples were withdrawn for assay of radioactivity at the times indicated. ◇——◇, control; □——□, + 50 ng per ml of dsRNA; ○——○, + 5 ng per ml of dsRNA; ●——●, + 0.5 ng per ml of dsRNA. *C*, 120 μl of a different lysate were preincubated at 30° with hemin and with the concentrations of f2 dsRNA indicated. At the times shown 20-μl samples from the preincubations were mixed with 5 μl of master mix and incubated for a further 30 min at 30° to determine [^{14}C]phenylalanine incorporation. The values for these incorporations are plotted against the length of preincubation. ○——○, control; ■——■, + 5 ng per ml of dsRNA; ●——●, + 1 ng per ml of dsRNA; △——△, + 0.6 ng per ml of dsRNA; □——□, + 0.3 ng per ml of dsRNA; ◇——◇, + 0.08 ng per ml of dsRNA.

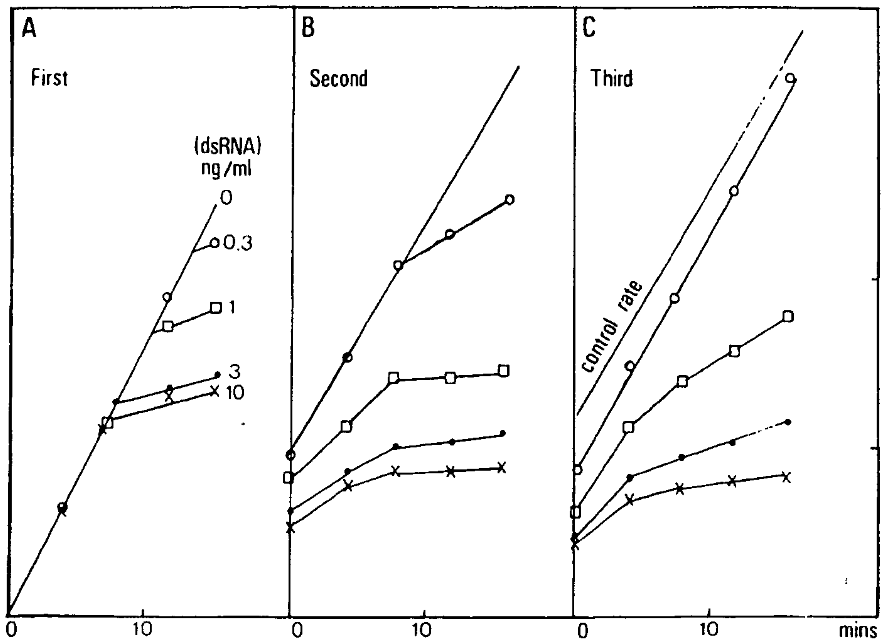
different types of dsRNA we found the same shape of curve in each case.

At high concentrations of dsRNA, when no inhibition was evident, equal amounts of normal α and β chains were being synthesized. During the latent phase, with inhibitory concentrations of dsRNA, the ratio of α and β chains synthesized was also unity. However, during the inhibitory phase, when the synthesis of both chains was markedly reduced, the residual synthesis represented an approximately 2-fold excess of β chain synthesis over α chains (data not shown). This finding may reflect the higher affinity of the β chain mRNA for the few remaining active ribosomes (28).

Effect of Preincubation of Lysates with dsRNA—The inhibition described in the previous section was observed at all temperatures between 20 and 37°, but the level of incorporation reached before onset of inhibition decreased as the temperature was lowered. This observation prompted us to test whether the length of the latent phase was changed if the lysates were preincubated with hemin and dsRNA in the absence of the other components necessary for protein synthesis. Preincubation for up to 1 hour under these conditions without dsRNA only marginally reduced the capacity of the lysates to synthesize protein (see Fig. 3*C*), nor were such preincubated lysates significantly more sensitive to dsRNA when this was added after the preincubation (data not shown). In contrast, when the dsRNA was present in the preincubation, the lag before onset of inhibition was greatly reduced by comparison with the lag observed if the same concentration of dsRNA was added at the start of a normal incubation (*cf.* Fig. 3*A* with Fig. 3*B*). At all concentrations tested, preincubation with dsRNA increased the degree of inhibition in a manner directly related to the length of the preincubation (Fig. 3*C*). With the higher concentrations of dsRNA the inhibition reached a maximal level such that the residual incorporation probably represented run-off of the ribosomes from the pre-existing polysomes. The increase in sensitivity obtained with preincubation enabled us to detect concentrations of dsRNA as low as 0.1 ng per ml (Fig. 3*C*). The contents of Table I show that only RNAs which would normally be classified as double-stranded show this potentiation of inhibition upon preincubation.

Evidence for Formation of Inhibitor in Lysates Incubated with dsRNA

Further experiments were designed to distinguish between the two distinct ways in which dsRNA could cause inhibition: (*a*) some component in the lysate essential for initiation may be effectively removed from the system by binding to dsRNA or by



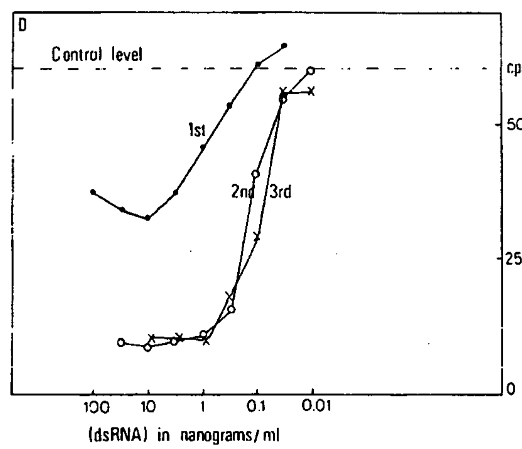


FIG. 4. Detection of an inhibitor in lysates incubated with dsRNA. *A*, 10-μl aliquots were withdrawn at the times shown from 100-μl incubations set up as described under "Incubation Conditions" with the following concentrations of *Penicillium chrysogenum* dsRNA: 100, 30, 10, 3, 1, 0.3, 0.1, 0.03, and 0.01 ng per ml. Kinetics of amino acid incorporation into protein (cpm) are shown only for a control and the following concentrations of dsRNA: ——, control; ×——×, 10 ng per ml of dsRNA; ●——●, 3 ng per ml of dsRNA; □——□, 1 ng per ml of dsRNA; ○——○, 0.3 ng per ml of dsRNA. *B*, after 16 min, the incubations described in *A* were cooled in ice and 1 volume (30 μl) was mixed with 2 volumes of fresh incubation mixture containing all the components described under "Incubation Conditions," including hemin and radioactive amino acid, thus generating 3-fold diluted incubations containing final concentrations of dsRNA of 33 ng per ml etc. These mixtures were then incubated and 10-μl samples were withdrawn at the times shown for assay of radioactivity. Final dsRNA concentrations were as follows: ——, control (no dsRNA); ×——×, 3.3 ng per ml of dsRNA; ●——●, 1 ng per ml of dsRNA; □——□, 0.33 ng per ml of dsRNA; ○——○, 0.1 ng per ml of dsRNA. *C*, after 16 min the incubations described in *B* were cooled in ice and a 3-fold dilution was carried out as described in *B*. These new mixtures were then incubated and 10 μl samples were taken at the times shown for assay of radioactivity. Final dsRNA concentrations were as follows: ——, control (no dsRNA); ×——×, 1.1 ng per ml of dsRNA; ●——●, 0.33 ng per ml of dsRNA; □——□, 0.11 ng per ml of dsRNA; ○——○, 0.033 ng per ml of dsRNA. *D*, the results of *A*, *B*, and *C* together with the parallel incubations containing the other dsRNA concentrations described in *A* but not illustrated, are replotted to show the net synthesis occurring in each of the three 16-min incubation periods against the actual concentrations of dsRNA in the incubations on a logarithmic scale. ●——●, data from the first incubation (*A*); ○——○, data from the second incubation (*B*); ×——×, data from the third incubation (*C*). - - -, mean value of the three control incubations (*i.e.* those lacking dsRNA) which differed by less than 10%.

being inactivated in the presence of dsRNA, or (*b*) the presence of dsRNA in the incubation may lead to the formation of an initiation inhibitor, such as occurs when lysates are incubated without hemin (14, 29). This type of inhibitor may be detected by mixing an inhibited lysate (*i.e.* one that has been incubated with dsRNA under standard conditions for protein synthesis assays) with a portion of fresh lysate and testing whether the protein synthesis activity of this mixture is more severely restricted than would be expected from a consideration of the amount of dsRNA present. An experiment of this type is illustrated in Fig. 4. First, standard protein synthesis assays were carried out with various concentrations of dsRNA (Fig. 4*A*). After 16-min incubation, 1 volume of each of these assays was mixed with 2 volumes of fresh incubation mix containing unincubated lysate and all the usual components for protein synthesis assays, but no dsRNA. Protein synthesis in these mixtures was then monitored over a 16-min incubation period (Fig. 4*B*), and then each assay was diluted again 3-fold in fresh incubation mix and incorporation assayed for another 16 min (Fig. 4*C*). The total synthesis occurring in the 16 min incubation period is plotted against the actual concentration of dsRNA in each incubation (Fig. 4*D*). The synthesis occurring in the second incubation (Fig. 4*B*) (where the dsRNA concentration had been reduced 3-fold) is much less than would be expected by comparison with the synthesis occurring in the first incubation (Fig. 4*A*) even allowing for the possibility that the ribosomes in the portion of the lysate previously incubated with dsRNA might be irreversibly inactivated. We also verified that the characteristics of inhibition were the same in the first, second and third incubations, namely loss of polysomes and loss of Met-tRNA$_f^{Met}$ from the 40 S ribosomal subunit.

The detection of the induced inhibitor in the assays described

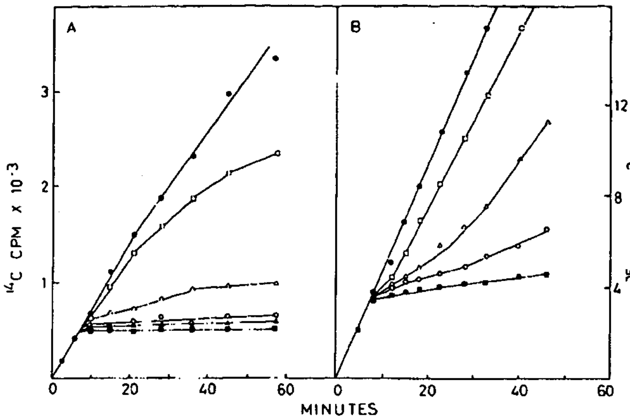


FIG. 5. The addition of high levels of dsRNA to a lysate incubated with low levels of dsRNA. *A*, incubations were set up as described under "Incubation Conditions" either without added dsRNA or with 10 ng per ml of *Penicillium chrysogenum* dsRNA. Samples (15 μl) were withdrawn at the times shown to assay for radioactivity. Aliquots (150 μl) were taken from the incubation containing 10 ng per ml of dsRNA at 1, 2, 3, 4, 5, 7, and 10 min after the start of the incubation and the concentration of dsRNA was raised to 10 μg per ml by addition of $1/100$ volume of a 1 mg per ml of *Penicillium chrysogenum* dsRNA solution, before continuing the incubation. The time courses for the incubations where the concentration of dsRNA was raised to 10 μg per ml at 1, 2, 3, and 4 min after the start of the incubation are shown. ●——●, control; ■——■, 10 ng per ml of dsRNA throughout; □——□, 10 μg per ml of dsRNA added at 1 min; △——△, 10 μg per ml of dsRNA added at 2 min; ○——○, 10 μg per ml of dsRNA added at 3 min; ▲——▲, 10 μg per ml of dsRNA added at 4 min. *B*, another set of incubations with a different lysate were set up as described in *A*, with the following differences. The incubations contained [^{35}S]methionine and 5-μl samples were taken for estimation of radioactivity incorporated into protein. In this case the concentration of dsRNA was raised to 10 μg per ml at 5, 8, and 15 min after the start of the initial incubation. ●——●, control; ■——■, 10 ng per ml of dsRNA throughout; □——□, 10 μg per ml of dsRNA added at 5 min; △——△, 10 μg per ml of dsRNA added at 8 min; ○——○, 10 μg per ml of dsRNA added at 15 min.

above is based on the fact that protein synthesis in a mixture of fresh lysate and lysate previously incubated with dsRNA was considerably less than would be expected from the concentration of dsRNA present. This marked difference between the observed and expected protein synthesis was observed only if the lysate which had been incubated with dsRNA was moderately diluted (2- to 4-fold). If the dilution was more extensive (*e.g.* 10-fold) then the difference was barely detectable.

In an attempt to establish an assay for the induced inhibitor which was not dependent on a comparison between observed and expected levels of protein synthesis, we tested the effects of adding noninhibitory high concentrations of dsRNA at various times to lysates incubated with a low level of dsRNA. This experiment produced two types of result; the variability appeared to depend on the particular reticulocyte lysate used. One type of response was that the inhibitory effects of the low level of dsRNA became completely dominant over the noninhibitory effects of 10 μg per ml of dsRNA after a few minutes, and several minutes before the inhibition of protein synthesis in the assays containing low concentrations of dsRNA was manifest (Fig. 5*A*). The other response observed was that a lysate incubated with low concentrations of dsRNA could be reactivated by the addition of 10 μg per ml of dsRNA even if this was added after the cessation of protein synthesis (Fig. 5*B*). However, this reactivation was never complete and never occurred immediately on adding the high concentration of dsRNA; the longer the lysate had been incubated with a low concentration of dsRNA before the addition of the high concentration the longer the lag and the less complete the reactivation.

Specificity—Table I lists all the nucleic acids we have tested for inhibitory activity in the reticulocyte lysate. We have divided them into three classes as follows (*a*) those displaying inhibitory kinetics characteristic of dsRNA; (*b*) those displaying inhibitory kinetics characteristic of single-stranded homopolynucleotides; (*c*) those nucleic acids causing no inhibition of protein synthesis at any concentration up to the highest tested.

The lowest concentration to give detectable inhibition is listed for each nucleic acid in Class *a*. The concentration giving maximum inhibition is shown for those cases where this was tested and these values are clearly all of the same order of magnitude. It did not seem that natural dsRNAs were better inhibitors than synthetic dsRNAs except in the case of poly(G)·(C) which might reflect a slight variation in the helix structure. It is clear that there is very little specificity with regard to base composition or base sequence in the inhibition of protein synthesis. Note that poly(A-U), an alternating copolymer which forms hairpin-like structures, is a perfectly good inhibitor of the dsRNA type. The presence of "tails" on the replicative ensemble of f2 did not alter its effectiveness as an inhibitor if a correction was made for the percentage of the RNA which was single stranded. Surprising inclusions in Class *a* are RNAs isolated from highly purified preparations of Qβ, f2, and R17 phages. However, it has already been shown by other methods that Qβ phage contains up to 2% dsRNA (24). We have been able to show that f2 and R17 phages also contain appreciable if somewhat lower amounts of dsRNA (Table I) (19). For all three phage RNAs it is possible to separate the inhibitory dsRNA fraction from the 28 S viral RNA by sucrose density gradient centrifugation (19). Purified 28 S viral RNA did not exhibit inhibition of a dsRNA type, and instead, if high enough concentrations were used, inhibition of a ssRNA type was seen, as other workers have noted (30). In Class *b* the lowest concentration to give detectable inhibition is listed. All these nucleic acids were tested under conditions of preincubation, but none of them showed enhancement of inhibition. For Class *c* the highest concentration tested is listed, although a whole range of concentrations was tested to ensure that an inhibition of the dsRNA type was not being masked by concentration dependence. All the nucleic acids in Class *c* were tested in the preincubation assay, but still showed no inhibition. In the case of the f1 RNA-DNA hybrids, an inhibition of the dsRNA type was observed at high concentrations. However, this inhibitory activity was not susceptible to pancreatic DNase digestion and we feel that this inhibition was due to a contamination of the hybrid preparation with dsRNA generated during the synthesis of the hybrid with *E. coli* RNA polymerase (see "Preparation of Polynucleotides"). Note that despite the common sequences shared by all the polynucleotides derived from f1 phage, only f1 dsRNA is inhibitory.

Size Requirement—To determine the minimum size of dsRNA effective in inhibition, a preparation of *Penicillium chrysogenum* dsRNA containing a major 14 S species and a minor fraction of smaller dsRNA molecules of random size was fractionated on a sucrose gradient (Fig. 6). Each fraction was tested for inhibitory activity at the same final RNA concentration (10 ng per ml) and the percentage inhibition caused by each fraction is plotted in Fig. 6. When RNAs from selected regions of the original gradient were rerun, they sedimented with approximately their original S value as judged both by optical density and by inhibitory activity. This indicates that the distribution of dsRNA on the

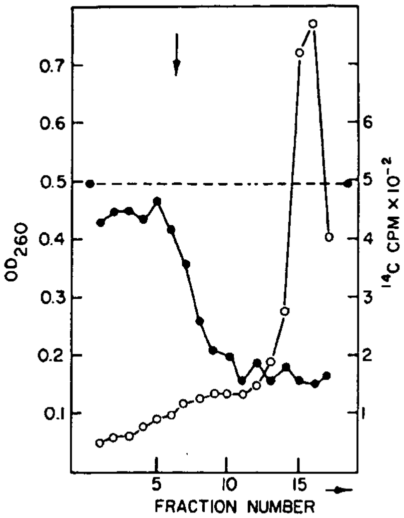


FIG. 6. Sizing of inhibitory dsRNA. *Penicillium chrysogenum* dsRNA (250 μg) was layered in a volume of 100 μl onto a 5-ml linear 5 to 20% sucrose gradient in 70 mM KCl, 1 mM $MgCl_2$, and 10 mM Tris-HCl (pH 7.2), and centrifuged for 10 hours at 36,000 rpm at 2° in a Beckman SW-50 rotor. The gradient was fractionated and the optical density at 260 nm of each sample was determined (O——O). Sedimentation was from left to right. Dilutions were made of each fraction so that they contained equal weights of dsRNA. These dilutions were then assayed at a final concentration of 50 ng per ml for their inhibitory activity as described under "Incubation Conditions." All fractions showed inhibitory kinetics typical of dsRNA. The radioactivity incorporated in 40 min at 30° is shown (●——●) together with a control value (●- - -●) at 40 min. In the cases where inhibition was seen, this value corresponds to the plateau value. The *arrow* indicates the position of the peak of *Escherichia coli* tRNA run in a parallel gradient.

initial gradient was a true reflection of the sizes of dsRNA present in our preparation of *Penicillium chrysogenum* dsRNA. The cut-off of effective inhibition occurs at around 4 S as judged by the position of a tRNA marker in a parallel gradient. Using a relationship devised to determine molecular weight from S value for dsRNA (31), the minimum effective size for inhibition would appear to be about 50 base pairs.

In addition, exhaustive RNase III digests of dsRNA did not inhibit over a wide concentration range, even if preincubation was used. The size range of exhaustive RNase III digests has been shown to be 10 to 20 base pairs (32), although it is not known whether all this product exists in a base-paired form.

We have used the abolition of inhibition on predigestion with RNase III as a further diagnostic test for nucleic acids displaying dsRNA type inhibition. None of the dsRNAs tested in this manner showed inhibition after digestion with RNase III, whereas the inhibition observed with poly(U) was unaffected by predigestion with RNase III (Table I).

A further indication of the size limit for inhibition comes from the use of dsRNA prepared from *E. coli* infected with the sus3 mutant of f2. This RNA is about 30 to 50 base pairs in size, and while it acts as a good competitive inhibitor of RNase III,[2] it did not inhibit protein synthesis in the lysate.

Fate of dsRNA during Inhibition

In order to test the stability of RNA added to the lysate ^{32}P-labeled f2 dsRNA or ssRNA were added to the lysate at a concentration of 50 ng per ml. Samples were taken for acid precipitation at various times during a complete incubation of the lysate. The dsRNA was completely stable, while the ssRNA was slowly converted to acid solubility. Addition of RNase III at the end of the incubation, however, caused 90% loss of acid-insoluble radioactivity, showing that it was still in the form of dsRNA. We also tried to see if any replication of the added dsRNA occurred, by adding, in addition to the normal components, CTP and [α-^{32}P]UTP to the incubation. There was no incorporation of label into acid-insoluble material, thereby ruling out this possibility. (Lower limit of detection at 50 ng per ml of dsRNA was 1 ng per ml of RNA synthesized.)

^{32}P-labeled dsRNA was also used in an attempt to demonstrate an association between dsRNA and a ribosomal component. Neither with f2 [^{32}P]dsRNA (50 ng per ml; specific activity 2×10^8 cpm per μg) nor with f1 [^{32}P]dsRNA (2 ng per ml; specific activity 1×10^7 cpm per μg) were we able to detect any stable association of dsRNA with any of the ribosomal constituents by sucrose gradient analysis either during the latent or the inhibitory phase. In these experiments we also confirmed that at the onset of the inhibitory phase there was a dramatic breakdown of the polysomes together with loss of nascent chains (1) and an increase in the amount of ribosomal subunits (12). Although negative, these experiments were sensitive enough to have detected a stable interaction of dsRNA with the ribosomes at a level of 0.1 ng per ml, the lowest inhibitory concentration of dsRNA. They cannot rule out, however, a transient or unstable association of dsRNA with the ribosomes.

DISCUSSION

The initiation of protein synthesis in reticulocyte lysates is inhibited by several types of polynucleotides: dsRNA (2), single-stranded f2 RNA (30) and homopolynucleotides such as poly(I) (33). Our results show that the following criteria can unambiguously distinguish the inhibition caused by dsRNA from the effects of other polynucleotides: (*a*) the much lower concentrations of tions of dsRNA required for inhibition; (*b*) the failure of high concentrations of dsRNA to inhibit; (*c*) the biphasic kinetics of protein synthesis; (*d*) the disappearance of 40 S/Met-tRNA$_f^{met}$ complexes shortly before the abrupt cessation of synthesis (12) (in contrast poly(I) causes a large increase in the level of these complexes (16)); (*e*) the formation of a potent inhibitor of initiation in lysates incubated with dsRNA; (*f*) the loss of inhibition observed upon pretreatment of the dsRNA with *E. coli* RNase III (a nuclease specific for double-helical RNA (24)).

These differences indicate that dsRNA inhibits initiation by a different mechanism than do other polynucleotides. The stringent specificity for dsRNA coupled with the lack of specificity for nucleotide sequence suggests that the inhibition involves interaction of the dsRNA with protein rather than nucleic acid. The requirements for inhibition are double-stranded RNA segments of at least 50 base pairs in length, but since the specific inhibitory activity does not vary much with the size of the dsRNA above this minimum length, the size of the binding site of the protein(s) is likely to be shorter than this. On the other hand the binding site seems likely to be greater than a single base pair, so that we can infer that a level of 0.6 ng per ml of dsRNA (10^{-9} M in base pairs), which is close to the minimum inhibitory concentration, would represent a concentration of protein binding sites of not more than 5×10^{-10} M, and possibly as little as 10^{-11} M. In contrast our lysates typically contain ribosomes at about 2.5×10^{-7} M (16) and native subunits at about 10^{-8} M (5% of the total ribosome population). If initiation factor proteins are present in approximately the same concentrations as the native

[2] H. D. Robertson, unpublished data.

subunits, which seems likely especially with factors that have anti-association factor activity, these considerations eliminate any models in which dsRNA inhibits initiation by irreversibly binding an initiation factor and effectively removing it from the system. Hypotheses in which the initiation factor binds to the dsRNA but is later released in an inactive form are still tenable, but such models would seem to suggest that addition of dsRNA at 100 to 1000 times the minimum inhibitory level (*i.e.* at levels where there would be enough binding sites to accommodate all the initiation factor present in the lysate) should cause almost instantaneous inhibition of initiation, rather than the slight reduction in the lag period which is in fact observed.

Those considerations lead us to question the hypothesis proposed by Kaempfer and Kaufman (34) that initiation is inhibited because IF-3, an initiation factor with anti-association factor activity (35), is inactivated by direct complexing with dsRNA. This hypothesis is based on the observations that supplementation of the lysate with IF-3 prevented the inhibition caused by dsRNA and that IF-3 itself bound strongly to dsRNA. Although IF-3 bound to DNA less strongly (34), the discrimination between DNA and dsRNA in this binding reaction is nowhere near as great as in protein synthesis assays (Table I).

Our results suggest that the mechanism of action of dsRNA is more complex than direct inactivation of IF-3. We have shown that lysates which have been incubated with dsRNA contain an inhibitor more potent than the dsRNA itself, and all our observations are consistent with the idea that the effect of dsRNA on protein synthesis is explicable in terms of the formation and action of this inhibitor. We do not as yet know the nature of this inhibitor other than that gel filtration studies have shown it to be macromolecular.[3] It is not impossible that interaction of IF-3 with dsRNA is part of the inhibitor-forming process. It is also possible that the inhibitor inactivates IF-3, although we favor a catalytic rather than stoichiometric relationship between the two.

Since the addition of high levels of dsRNA to lysates incubated with inhibitory concentrations for various times produced two different types of response, depending on the particular lysate used, it seems necessary to postulate that the inhibitor may exist in two possible forms; one reversible by high levels of dsRNA and one irreversible. The results in Fig. 5 suggest that in some lysates the irreversible species is formed significantly more slowly than the reversible species, whereas in other cases the irreversible form is generated very rapidly. Although the existence of two forms of inhibitor and the variability between lysates are puzzling features, they are not unprecedented. There is a close parallel between lysates incubated with low levels of dsRNA and those incubated without hemin. In both situations the kinetics of protein synthesis are biphasic (17, 36) and the 40S/Met-tRNA$_f^{Met}$ complexes disappear shortly before the abrupt cessation of protein synthesis (29). In addition we have shown elsewhere that a number of compounds can prevent and reverse the inhibition seen in both conditions (13), whereas Kaempfer and Kaufman have shown that addition of excess IF-3 prevents not only the inhibition caused by dsRNA (34) but also that caused by omission of hemin (35). The two types of response, no reactivation or delayed reactivation, noted in Fig. 5, are also seen when hemin is added to lysates incubated for various times without hemin (36, 37). Several lines of evidence have led to the conclusion that incubation without hemin leads to the formation of an inhibitor which may exist in two forms, reversible by hemin or irreversible (29, 37). The reversible species may be a precursor of the irreversible form (37), which has been partially characterized as a large molecule and which may act catalytically since one molecule can apparently prevent the functioning of several hundred ribosomes (38). A further similarity between lysates incubated with low levels of dsRNA or without hemin is that they are committed to stop protein synthesis several minutes (and therefore several rounds of synthesis) before inhibition actually occurs, since the addition of the appropriate reactivating substance after a brief incubation period does not prevent the onset of inhibition although it may render the shut-off transitory (Fig. 5 and Refs. 17, 36, and 37).

In spite of the close similarities described, we do not wish to imply that the mechanism of inhibition of protein synthesis is absolutely identical in the two cases. If this were so, we might expect that high levels of dsRNA could substitute for hemin in preventing inhibition, and we have so far failed to observe this. Also, preliminary gel filtration studies suggest that the inhibitor formed in the presence of dsRNA is larger than that formed in the absence of hemin. It seems clear that in both situations the inhibition involves a complex multi-step process. It is possible that the terminal stages of this process are identical in the two conditions, whereas the early steps are different.

[3] Unpublished observations.

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